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Molecular Detection of *Ehrlichia phagocytophila* Genogroup Organisms in Larvae of *Neotrombicula autumnalis* (Acari: Trombiculidae) Captured in Spain

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ABSTRACT: Twenty unfed larvae of *Neotrombicula autumnalis* (Acari: Trombiculidae) collected on vegetation in the north of Spain were examined by polymerase chain reaction for *Borrelia burgdorferi* (s.l.), rickettsiae, and the *Ehrlichia phagocytophila* genogroup. At least 10% of the larvae were found to contain granulocytic ehrlichiae. Because the larvae were unfed, they would necessarily have inherited the bacteria through a transovarian transmission pathway.

The trombiculids are mites of medical and veterinary importance owing to their troublesome bites on animals and humans and their role as vectors of *Orientia tsutsugamushi* (scrub typhus) in Asia and other parts of the eastern hemisphere. In Europe, the Trombiculidae have not been reported to be vectors of diseases, and only a few species, such as *Neotrombicula autumnalis,* are known for their irritating bites. Here, it is reported for the first time that the Trombiculidae, at least *N. autumnalis,* are carriers of members of the *Ehrlichia phagocytophila* genogroup, organisms that to date were believed to be transmitted only by ticks and that are of increasing importance in relation to the human granulocytic ehrlichiosis as an emerging infectious disease (Daszak et al., 2000).

Two different samples of mites were studied. Sample A consisted of 20 specimens collected on vegetation between November and December 1999 in a mountainous zone (Puerto Piqueras) of the province of Soria (Spain). These mites were submitted in 70% alcohol to our laboratory for identification and to determine if they might be responsible for bites that occur of people during the autumn months in that area. All specimens were identified as unfed larvae of *N. autumnalis* (Fig. 1) and subsequently they were randomly divided in 2 halves. One-half was stored and the other was subjected to polymerase chain reaction (PCR) analysis to check whether they were carriers of any of the tick-borne pathogens routinely checked for in ticks removed from humans, i.e., *Borrelia burgdorferi* sensu lato, spotted fever group rickettsiae, and the *E. phagocytophila* genogroup. Sample B consisted of 30 specimens of Trombiculidae (*Neotrombicula* sp.) collected in December 1999 from 4 house and wild mice captured in the urban and periurban area of a small agricultural town in the province of Salamanca (Spain) free of domestic ruminants. The larvae were in different degrees of engorgement and, after being soaked once in 70% alcohol and 3 times in ultrapure water, they were stored at −20 C until processing.

To extract DNA, the larvae from sample A were first sequentially washed in 45% alcohol, 30% alcohol, and ultrapure water, and then crushed with sterile tweezers under a binocular lens. The fragments were transferred to a test tube and DNA was extracted in 100 μl of 5% Chelex (Bio-Rad, Hercules, California) following the procedure already described by Guttman et al. (1996). The specimens of sample B were fragmented while frozen, pooled, and their DNA was also extracted in 100 μl of 5% Chelex.

These DNA samples were used as templates in PCR analyses to determine the presence of the organisms being studied. Amplification of the target sequences was carried out with the primers and conditions previously described for *B. burgdorferi* s.l. (Postic et al., 1994), *Rickettsia* sp. (Regnery et al., 1991), spotted fever group rickettsiae (Roux et al., 1996), and the *E. phagocytophila* genogroup (Goodman et al., 1996; Chang et al., 1998). Positive and negative controls were included in all PCR runs. The positive controls were, respectively, DNA from *B. burgdorferi* strain Esp-1, from *R. connori,* and from *E. phagocytophila.* As negative controls, both ultrapure water and DNA from ticks free of the above pathogens were incorporated in each run. PCR results were analyzed, depending on the size of the expected band, in 1% or 2% agarose gels containing ethidium bromide. The DNA from positive amplifications was purified (QIAquick PCR Purification Kit, QIAGEN, Hilden, Germany) and sequenced in a fluorescence-based automated sequencing system (ABI 377 DNA Sequencer; Perkin-Elmer Instruments, Norwalk, Connecticut) with the same primer set used in the amplification. The sample DNA extraction, the amplification, and the analysis of the PCR product were each performed in different rooms to prevent contamination. The sequences generated were compared against those available in standard databases (GenBank, EMBL, DDBJ) using the BLAST 2.0 search program.

The first half of sample A was negative for *B. burgdorferi* and rickettsiae, but positive for the *E. phagocytophila* genogroup, as indicated by the presence of a 151-base-pair (bp) band (Fig. 2). Since Trombiculidae have not been reported to be vectors of ehrlichiae, to confirm this finding we analyzed the second half of sample A. Because the specimens had been taken in a random fashion, it was surmised that if the first half was positive, then the second half would also be positive, except if only 1 specimen in the whole sample was infected (an unlikely possibility since all mites came from the same source). The second half was processed in the same way as was the first, but in a different building and with different instruments to avoid contamination. As expected, the second half also possesses a 151-bp band for the *E. phagocytophila* genogroup. The nucleotide sequences of both amplicons were identical and showed 100% identity with the corresponding 151-bp segment of the 16S rRNA gene shared by *E. phagocytophila* (GenBank accession M73220), *E. equi* (M73223), and the HGE agent (U02521). To strength-

FIGURE 1. *Neotrombicula autumnalis* larva from the sample A (captured in Puerto Piqueras, Soria, Spain).

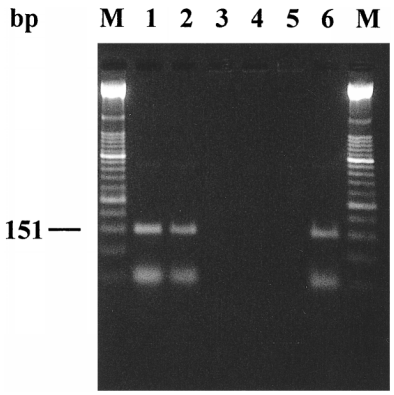

FIGURE 2. Ethidium bromide-stained 2% agarose gel showing the 151-base-pair polymerase chain reaction products obtained with the specific primer set GER3 and GER4 for the *Ehrlichia phagocytophila* genogroup. Lanes 1 and 2, first and second halves of sample A; 3, sample B; 4, negative control (distilled water); 5, negative control (tick DNA); 6, positive control (*E. phagocytophila* DNA); M, molecular size marker XIII (Boerhinger Mannheim, Mannheim, Germany).

en these findings, both halves of sample A were reanalyzed 2 more times, giving the same results.

Sample B was negative for all the pathogens tested. On repeating the analyses, the same negative results were obtained.

In trombiculid mites, the adults and nymphs are free-living organisms and only the larvae are parasites. It is important to note that the presence of the *E. phagocytophila* genogroup is not a transient event owing to the feeding of the mites on a carrier animal since all the larvae were collected unfed on vegetation. It can also be ruled out that the results could have been due to contamination during the handling of the samples, because the negative controls were never amplified, or to nonspecific amplifications, because in this latter case sample B would also have been positive. Because the specimens of sample B came from an urban setting, it would have been highly unlikely that the populations present in that place could have entered into contact with animals harboring the bacterium; accordingly, it is not surprising that they were free of ehrlichiae. In the light of the above, it is believed that if the unfed larvae of sample A were positive, it was because they were true carriers of the bacteria that would necessarily have been inherited through a transovarian transmission pathway. Because the life cycle of *N. autumnalis* in Europe lasts 1 yr, one could speculate that in the places where it is present it could play as important a role as that of hard ticks, in which only transstadial transmission occurs (Piesman and Gage, 1996). The high proportion of infected specimens in the sample analyzed (at least 10%) supports this idea.

These findings could be of great help in the diagnosis of human granulocytic ehrlichiosis in patients who do not lead physicians to suspect the disease because they had not been bitten by a tick and also in the diagnosis of ehrlichiosis in livestock during the months when ticks are scarce, such as November to January.

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